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13. ABSTRACT ***(Maximum 200 Words)***

Three pertinent novel observations were made in this grant period that have far reaching implications:
First, using tumor cells that secrete a genetically engineered form of a heat shock protein, gp96Ig, we showed that CD8 cells expanded and generated specific memory *in vivo* without the need for CD4 help. We have now analyzed this observation quantitatively by adoptive transfer of ovalbumin-TCR transgenic cells (OT1) into syngeneic mice as indicator cells *in vivo* and the use of EG7-gp96Ig, a tumor secreting gp96Ig and containing ovalbumin as surrogate antigen. Using k^b-tetramer assays, an expansion of OT1 is observed from an initial frequency of 0.5% to over 50% of all CD8 cells following primary injection and one boost with gp96Ig secreting, but not wild type EG7. Gp96Ig was found to directly activate dendritic cells without CD4 help. *Second*, this dramatic expansion of OT1 is seen in wild type mice, but it is completely abrogated in perforin deficient mice (PKO) and in perforin/Fas-ligand double deficient mice. Fas-ligand single deficient mice show normal expansion. This observation defines a novel role for perforin in CD8 activation and expansion via heat shock protein gp96 and its chaperoned peptides. *Third*, the dramatic expansion of cognate CD8 CTL is preceded by an equally dramatic expansion of NK cells that is also dependent on the presence of perforin.
In addition to the novelty and unexpected nature of these observations stimulating new research approaches, the data provide a firm basis for future clinical trials in several tumors, including breast tumors, using gp96-Ig based vaccines.

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Table of Contents

Introduction:

We have engineered the endoplasmic reticulum resident heat shock protein gp96 to delete the KDEL retention signal and replace it with the Fc region of IgG1. Because gp96 has previously been shown to chaperone peptides and to be immunogenic when isolated from tumor cells, we hypothesized that secreted gp96-Ig may carry along bound peptides. When secreted from tumor cells gp96-Ig would induce an immune response to the tumor by activation of CD8 cells. The work was designed and planned to test whether CTL activation could be achieved, and whether CTL activation was antigen specific.

The results indicate: *i.* that antigen specific CTL activation by secreted gp96-Ig can be achieved in vivo; *ii.* that this activation results in dramatic T cell expansion; *iii.* that T cell expansion can lead to tumor rejection and is therapeutically useful; and *iv.* that unexpected molecular mechanisms, involving perforin are participating in both NK and T cell expansion.

Body:

Previous data

Heat shock proteins can control CD8 CTL expansion. Several heat shock proteins carrying bound (chaperoned) peptides are bound by the major endocytic receptor CD91 (the α_2-macroglobulin receptor) on dendritic cells and macrophages (1, 2). After endocytosis by dendritic cells or macrophages, the heat shock protein-chaperoned peptides are channeled into the class-I MHC presentation pathway where they are powerful activators of cognate CD8 CTL (3, 4). Binding of heat shock proteins to CD91 and endocytosis serves as a novel maturation signal for dendritic cells which is independent of CD40-L/CD40 interaction (5). Based on this information, *in vivo* secreted, peptide-carrying heat shock proteins were predicted to activate cognate CD8 CTL without CD4 help, *in vivo*. As reported previously, this was confirmed in a tumor model using the ovalbumin transfected EL4 lymphoma line, EG7. The endoplasmic reticulum (ER) resident heat shock protein gp96 is normally retained in the ER through its C-terminal KDEL sequence. Replacement of gp96's KDEL with the Fc-portion of IgG1 and its transfection into EG7 (EG7-gp96Ig) results in secretion of soluble gp96Ig, together with peptides chaperoned by the heat shock protein. EG7 are highly tumorigenic and irradiated EG7 do not induce protective tumor immunity. In contrast, EG7-gp96Ig secreting the soluble heat shock protein with its chaperoned peptides (including ovalbumin derived), are rejected and induce protective and specific tumor immunity (6) (manuscript appended). EG7-gp96Ig rejection is dependent CD8 cells in both the afferent and efferent phase of the immune response. Depletion of CD4 cells by antibody or use of CD4 k.o. mice has no effect on rejection, while CD8 depletion results in tumor growth of EG7-gp96Ig.

New data

Secreted heat shock protein gp96Ig: an analytic tool for quantitation of CD8 cell expansion and contraction. Secreted heat shock protein gp96Ig generates a tumor rejecting and tumor protective immune response *in vivo* in syngeneic mice (6). EG7, an H2b tumor cell generated by ovalbumin transfection of the murine EL4 lymphoma, causes a 100% tumor incidence when transplanted at 10^5 cells or more s.c. Transfection of EG7 with gp96Ig, but not with vector alone, results in EG7-gp96Ig rejection by CD8 cells and generation of tumor immunity to the untransfected tumor EG7 and to EL4. Rejection is CD4 cell independent in both the afferent and efferent phase of the immune response, but requires CD8 cells.

These results suggested that *in vivo* secreted or released gp96 is a powerful immune stimulus for CD8 cells independent of concurrent CD4 help. Mechanistically we found that gp96Ig binds to the endocytosis-receptor CD91 on dendritic cells and macrophages. Endocytosis of CD91 is accompanied by DC maturation and by re-presentation of gp96Ig bound peptides by class I MHC on the dendritic cells, resulting in activation of cognate CD8 cells without CD4 help (5).

Quantitation of CD8 expansion and contraction. C57Bl/6 (B6) mice were injected i.v. with one million purified OT1 cells, isolated from C57Bl/6 ova-TCR ($k^{b\text{-}SIINFEKL}$ specific) transgenic splenocytes. Two days later the mice received one million EG7-gp96Ig intraperitoneally secreting gp96Ig at a rate of 50ng per 10^6 cells per 24 hours. At different

time intervals after injection of EG7-gp96Ig, samples were obtained from injected and from control mice (receiving untransfected EG7) and analyzed for the frequency of OT1 cells by tetramer analysis using k^b- tetramers (obtained from the NCI facility) loaded with SIINFEKL (ova-peptide).

Adoptive transfer of 1 million OT1 cells into w.t. B6 mice results in a frequency of 0.7±0.14% (SEM) of OT1 cells in the CD8 gate in peripheral blood or spleen (n =13). One week after i.p. injection of EG7-gp96Ig, the OT1 cells had expanded to 24.9±3.1% frequency of all CD8 cells. Without further boosting OT1 cell frequencies contracted in the second week to between 18.7±1.9% of the CD8 cells. A booster injection of EG7-gp96Ig on day 14 resulted in dramatic further expansion of OT1 to 49.5±3.7% frequency among all CD8 cells (Fig.1)

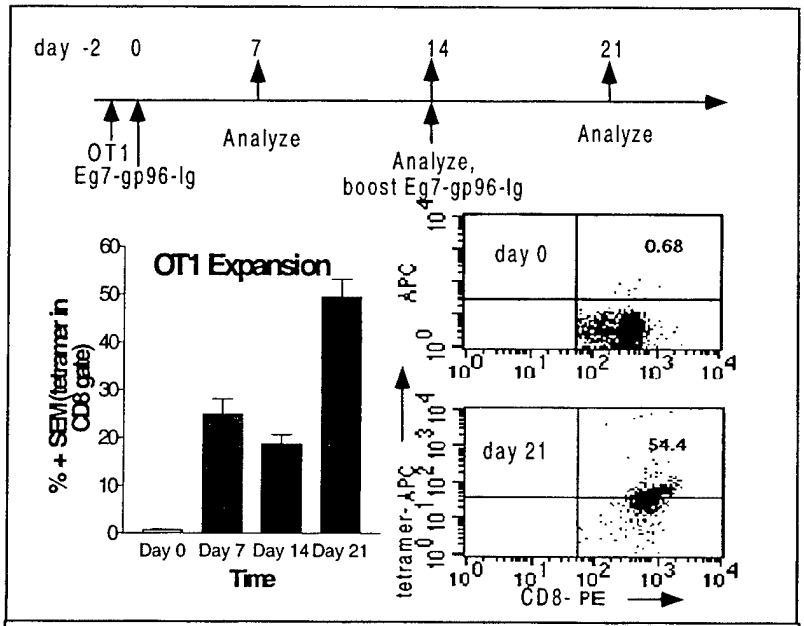

Figure 1: Expansion of OT1 in syngeneic B6 mice after vaccination with EG7-gp96Ig. The time line on top gives the sequence of treatments. 10^6 purified OT1 were injected i.v, followed on day 0 and 14 by 10^6 EG7-gp96Ig. Blood samples were obtained sequentially and analyzed The histogram shows the mean of the percentage of Kb-ova-tetramer positive cells (±SEM) within the CD8 gate. The right panels shows representative FACS plots on day 0 and day 21 after EG7-gp96Ig.

The results presented in Fig. 1 are highly reproducible and have been consistently observed in 13 mice in several experiments. Cognate CD8 (OT1) expansion was expected, because the secreted gp96Ig is known to carry ovalbumin derived peptides (6), however the extent of CD8 expansion by secreted gp96Ig was quite surprising. An expansion to ~25% after one immunization, and to 50% after one booster has not been reported for a vaccine (to the best of our knowledge), including peptide pulsed dendritic cells.

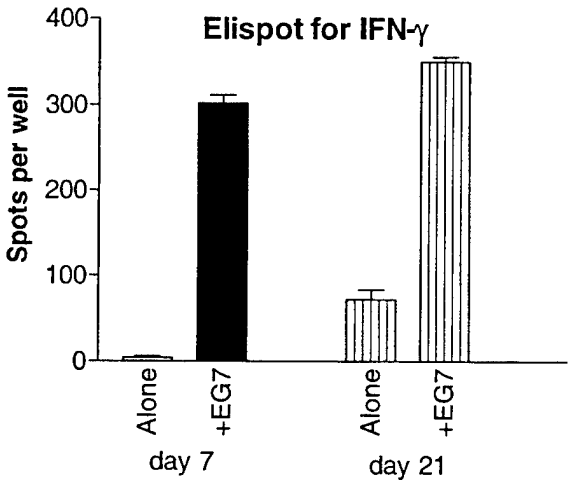

Figure 2: Expanded OT1 produce Ifn-γ upon stimulation in vitro. Conditions as in Fig.2, but spleen cells were analyzed on day 7 and day 21 post EG7-gp96Ig treatment of mice. $5x10^5$ spleen cells per well were incubated with $5x10^4$ irradiated EG7 for three days.

A comparable expansion of CD8 is seen only with some infectious agents, including LCMV and to a lesser extent Listeria monocytogenes, both of which infect monocyte/macrophages and cause strong CD8 CTL expansion (see above, background).

The expanded OT1 CTL are fully functional with regard to Ifn-γ production. In ELI-spot assays the increase in the number of Ifn-γ producing cells in response to EG7 was parallel to the increase of tetramer positive cells (fig. 2). It is evident that the ELIspot assay underestimates the frequency of tumor specific cells, probably because EG7 does not present ova peptide efficiently.

Gp96Ig is secreted by live tumor cells, EG7-gp96Ig. Although the cells divide, the rate of replication is minimal compared to viral replication. Unlike virus infection, EG7-gp96Ig do not cause tissue damage do not create an inflammatory stimulus after transplantation. However, the secretion of gp96Ig and its binding to CD91 on dendritic cells, causes DC maturation and CD8 CTL activation and expansion followed by rejection of the transplanted EG7-gp96Ig tumor cells.

Perforin is required for gp96Ig mediated CD8 CTL expansion. Having in hand a sensitive assay for CD8 CTL expansion and contraction *in vivo*, we studied the function of several genes in CD8 homeostasis. Evaluating the role of cytotoxic molecules, the

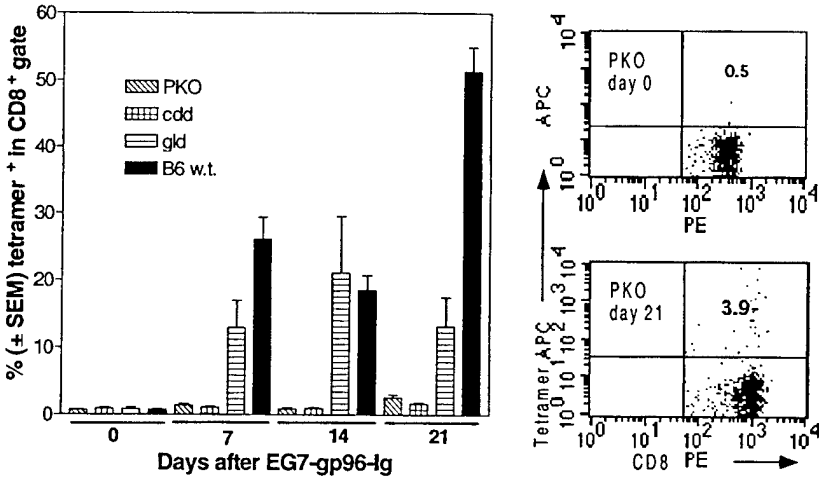

Figure 3: Perforin deficiency abolishes OT1 expansion in response to EG7-gp96Ig. PKO (n = 7) perforin deficient; cdd (n = 7) perforin/Fas-ligand double deficient; gld (n = 2) Fas-ligand defective; B6 w.t. (n = 7) wilt type C57Bl/6. The two right panels show FACS dot plots of APC (tetramer) positive cells within the CD8 gate and on day 0 and day 21 post EG7-gp96Ig in PKO mice.

expansion of OT1 cells by gp96Ig was measured in syngeneic (B6) perforin deficient mice (PKO), in Fas-Ligand defective (gld) mice and in Fas-L/perforin, cytotoxic double deficient (cdd) mice. Surprisingly, the absence of perforin completely abolished OT1 expansion (Fig. 3). In PKO mice and in cdd (perforin/Fas-ligand double deficient) mice OT1 did not expand in response to EG7-gp96Ig, while gld mice showed comparable expansion to wild type B6 mice after 7 and 14 days (Fig.3).

The adoptive transfer of perforin-containing OT1 to perforin deficient mice could have caused an immune response to perforin in OT1. To exclude this possibility, the OT1 TCR transgene was bred into the PKO background to generate perforin deficient OT1 cells. Adoptive transfer of perforin deficient OT1 into perforin deficient mice and subsequent stimulation with EG7-gp96Ig did not restore OT1 expansion (data not shown), excluding the possibility of an immune response to perforin. In another test we measured the expansion of endogenous kb-SIINFEKL-tetramer reactive CD8 cells. EG7-

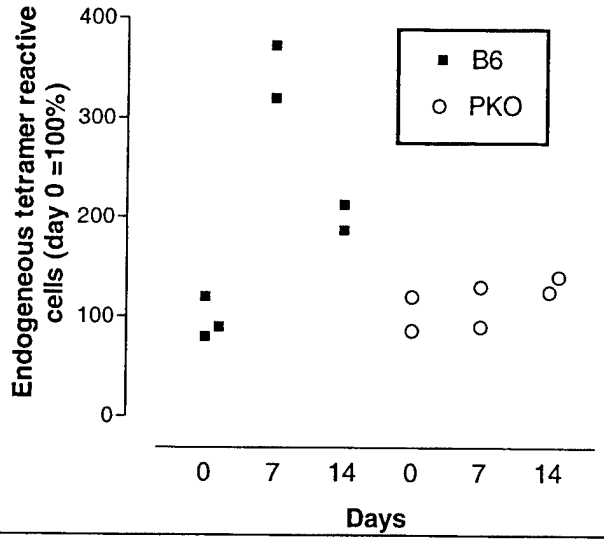

Figure 4: Perforin is required for expansion of endogenous K^{b}-$SIINFEKL$ reactive CD8 cells by EG7-gp96Ig. Normal B6 w.t. and Perforin deficient (PKO) mice received 10^{6} EG7-gp96Ig i.p. The frequency of tetramer positive cells was tested on day 0, 7 and 14 and is plotted relative to the frequency on day 0.

gp96Ig caused the 4-fold expansion of endogenous tetramer-reactive CD8 cells in wild type mice but not in PKO mice (Fig.4).

It is evident therefore, that perforin is required for CD8 CTL expansion *in vivo* in response gp96Ig stimulating maturation of dendritic. Fas-ligand is unable to substitute for perforin in PKO mice. Since neither perforin-containing nor perforin-deficient OT1 expand in PKO mice, it is also clear that it is not the perforin in the cognate CD8 CTL themselves, i.e. in OT1, that helps OT1 expansion in wild type mice. Cells providing perforin for OT1 expansion may be NK cells. While CD4 cells can also express perforin, they are less likely to be responsible for OT1 expansion based on our published finding that EG7gp96Ig can stimulate CD8 CTL mediated immunity without CD4 help (6).

LCMV also causes dramatic expansion of cognate CD8 CTL in wild type mice but also in perforin deficient mice (7-10). Badovinac et al found that absence of perforin enhanced the expansion of CD8 cells in a primary response to Listeria (11). On the other hand gp96Ig under the conditions described here causes comparable expansion of cognate CD8 CTL in wild type mice, but no expansion in perforin deficient mice. This unexpected difference points to an important role for perforin in CD8 expansion, presumably under conditions where there is minimal tissue damage and inflammation.

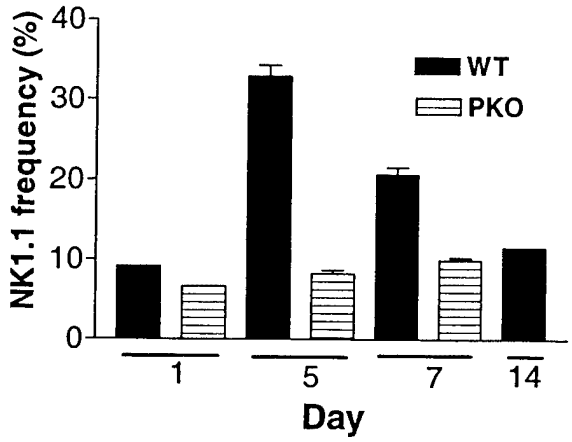

Figure 5: Expansion of NK cells upon gp96Ig vaccination in w.t. mice but not in perforin deficient mice. W.T. mice or PKO mice received 106 EG7-gp96Ig i.p. NK proliferation was measured in peripheral blood on day 1, 5, 7 and 14.

Tumor secreted Gp96-Ig causes NK cell expansion preceding CTL expansion Expnasion of NK cells was measured by flow cytometry using the NK1.1 antibody. NK cells upon vaccination with EG7-gp96Ig expanded from a base line of about 10% to more than 30% of all peripheral blood mononuclear cells within 5 days and subsequently declined up to day 14 (Fig. 5).

Interestingly, NK cell expansion also did not take place in the absence of perforin, indicating that perforin plays a critical role in NK and CTL activation by gp96.

Established tumors interfere with CD8 CTL expansion mediated by secreted gp96Ig. The dramatic expansion of cognate CD8 CTL in response to tumor secreted gp96Ig and the ability to quantitate the response allows a detailed investigation of the influence of established tumors on the CD8 CTL homeostasis. Conversely it is also possible with this methodology to ask how CD8 CTL frequency relates to tumor rejection and to elucidate genetic components required in the process.

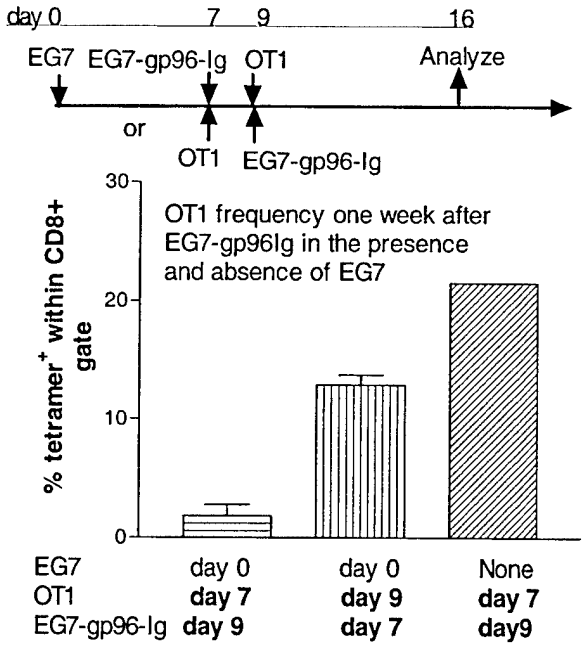

Figure 6: Exposure to pre-existing w.t.EG7 tumor interferes with OT1 expansion. Mice received 10^6 EG7 s.c. or no tumor. On day 7, 10^6 OT1 or EG7-gp96Ig were given, followed on day 9 by 10^6 EG7-gp96Ig or OT1, respectively. Seven days later OT1 expansion was measured in spleen and blood by tetramer staining.

The experimental model described above was modified in the following way. Wild type mice received one million EG7 (not-secreting gp96Ig) subcutaneously to allow tumor establishment for 7 days. On day 7 the mice received 1 million OT1 i.v.; two days later the mice were injected with 1 million EG7-gp-96-Ig. OT1 expansion was measured after additional seven days.

Not surprisingly, OT1 expansion was significantly diminished by the 7 day old EG7 tumor (Fig. 6, left column). To determine whether the diminished OT1 response was due to their presence for two days in the EG7 tumor-bearing mouse, prior to gp96Ig stimulation or whether the 7day tumor may have altered the response of dendritic cells to gp96Ig, the following variation of the experiment was done. EG7 was established for 9 days as above; then 1 million EG7gp96Ig was given i.p., followed on the same day by 1 million OT1 i.v. In this experiment OT1 are exposed concurrently to EG7 and to EG7-gp96Ig. Under these conditions OT1 expansion is restored almost completely (compared to no EG7) (Fig 6).

The results suggest that the presence of an established EG7 tumor, not secreting gp96Ig, is able to induce anergy in OT1 cells, thought to be due to lack of costimulation, in agreement with other reports (12-14). When EG7 and EG7-gp96Ig are present concurrently, anergy induction in OT1 is prevented and the OT1 become activated through secreted gp96Ig and its uptake by dendritic cells.

While induction of anergy in T cells by tumors is a well-documented phenomenon, it has not been easy to quantify in vivo. The experimental system outlined

above allows a detailed analysis and quantification of the induction of CD8 anergy in vivo. More importantly, the experimental system also allows analysis of mechanisms that may prevent anergy and block the establishment of tolerance to the tumor. Finally, the system is suitable for the determination of trafficking of in vivo activated CD8 CTL.

Successful tumor therapy with gp96-Ig vaccines requires multiple vaccinations. The induction of T cell anergy by tumors suggested attempts to break anergy by frequent vaccinations and boosting. As shown in in Fig 7 below, rejection of established tumors is

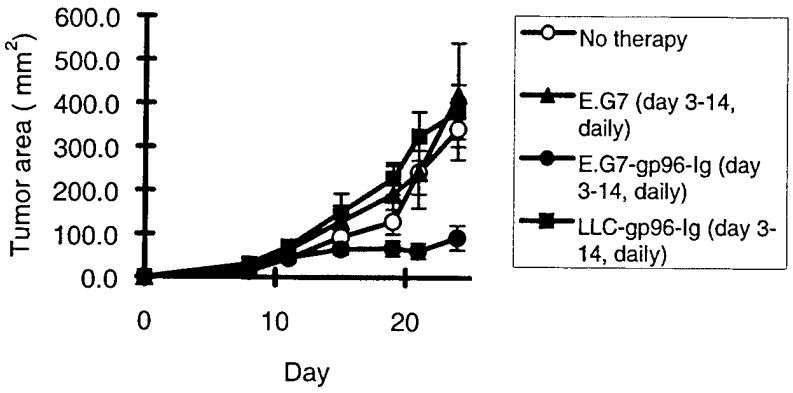

Figure 7: C57Bl/6 mice received $2x10^5$ EG7 tumor cells s.c. in the right flank. As vaccine 10^6 EG7-gp96Ig was injected s.c. in the left flank on the days indicated. Irradiated EG7 and LLC-gp96Ig served as controls for specificity. No treatment is hown in open circles.

possible when immunizations are carried out daily or even twice daily. Irradiated EG7 or LLC producing gp96Ig are ineffective

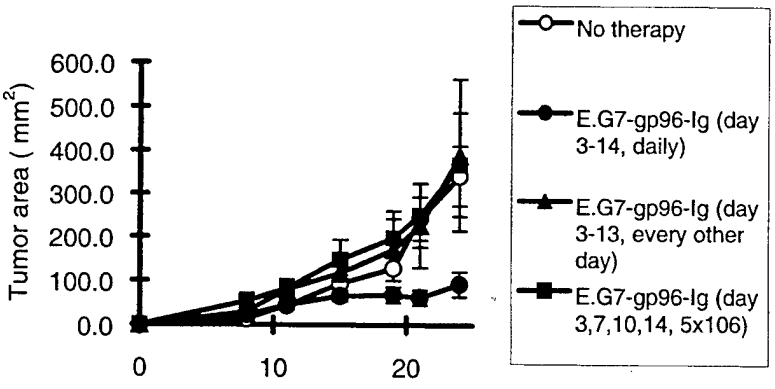

Figure 8: Immunization on alternating days is ineffective for therapeutic treatment of EG7. Experimental details are identical to Fig.7.

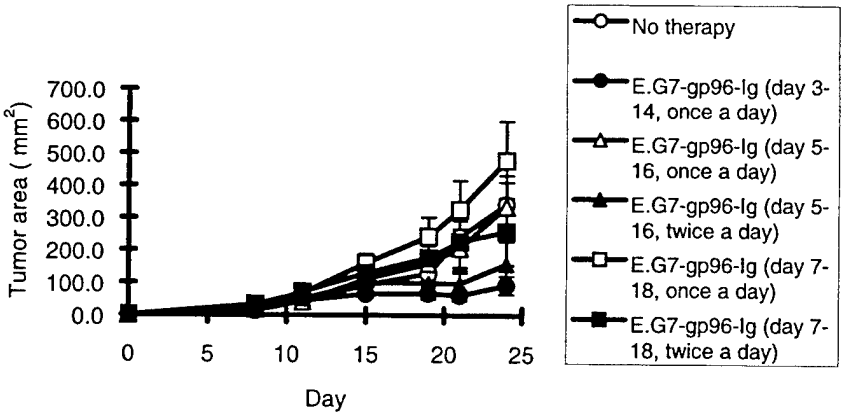

Figure 7: Therapeutic effect of twice daily vaccination with the gp96Ig producing tumor. Conditions of tumor inoculation and vaccination as in Fig.7 except for the dose schedule.

Even immunization every other day with EG7-gp96Ig is ineffective as shown in Fig. 8.

When immunization was carried out twice daily with 10^6 EG7-gp96Ig, Therapeutic effects were seen even when the tumor was allowed to establish itself for 5 or 7 days (Fig. 9). This is a remarkable finding, since the doubling time of EG7 is about 14 hours. It is extremely difficult to obtain therapeutic effects against this tumor once it is well established in vivo.

The therapeutic effect of the secreted heat shock protein could also be demonstrated in Lewis lung carcinoma (LLC) (fig. 10), a mouse tumor that is resistant to many forms of treatment.

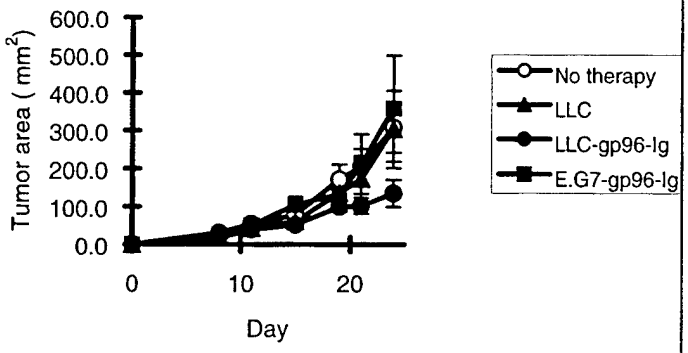

Figure 8: Therapeutic effect on LLC of LLC-gp96Ig vaccination. 10^5 LLC cells were implanted s.c. into the right flank of C57BL/6 mice on day 0 (n=10-13). Next, 1 X 10^6 irradiated LLC, irradiated LLC-gp96-Ig, E.G7-gp96-Ig or PBS were injected s.c. into the left flank of these mice on day 3, 7, 10 and 14. The speed of LLC growth was remarkably suppressed in the mice treated with LLC-gp96-Ig (statistically significant for almost all the observation periods), while control E.G7-gp96-Ig, LLC or PBS treated mice showed almost the same growth speed.

Key research accomplishments:

- Gp96 engineered to be secreted carries peptides
- Tumor secreted gp96-Ig is highly immunogenic and tumor specific
- Immunization by gp96-Ig depends on CD8 CTL and is independent of CD4 help in the afferent and efferent phase
- Immunization by gp96-Ig results in dramatic expansion of cognate CD8 CTL
- Frequencies of cognate CD8 CTL achieved upon gp96-Ig immunization are higher than observed in any other vaccine and resemble T cell expansion in certain viral infections
- Gp96-Ig vaccination is highly effective for tumor therapy
- Vaccinations need to be done daily or twice daily to be effective for rapidly growing tumors
- The mechanism of CD8 CTL stimulation by gp96-Ig relies on the activation of dendritic cells without CD4 help and channeling of the petide antigne to the class I MHC presentation pathway.
- CTL expansion mediated by gp96-Ig is dependent on the presence of perforin, but independent of Fas-Ligand

- Gp96-Ig vaccination also results in the dramatic expansion of NK cells, which also is dependent on the presence of perforin.

Reportable outcomes:

1. **Publications 1999 to 2001**

See reference (6, 15-32) in the reference list

2. **Abstracts**

CD40 independent priming of protective T cell immunity against tumor cells engineered to secrete a heat shock protein gp96
Jie Dai1, Diliana Stoilova1, Sreyashi Basu1, Robert J Binder1, Eckhard R Podack2 and Zihai Li1* (1st International Het Shock Conference, Farmington 1999)
Characterization of MHC restricted CTL for the isolation of tumor antigens
Koichi Yamazaki and Eckhard R .Podack (DOD Breast Cancer Meeting, Atlanta, 2000)
CD30 downregulates cytotoxic lymphocytes through several synergistic pathways
Hiromi Muta and Eckhard R. Podack (FASEB, San Francisco 2000)
Death receptor systems and Perforin in lymphocyte mediated cytotoxicity and homeostasis
Eckhard R. Podack (Neuro AIDS Program meeting, Miami, 2000)
Tumor Secreted Heat Shock-Fusion Protein Causes Rapid Expansion of Cognate TCR Transgenic CD8 CTL in vivo, which is Dependent on the Presence of Perforin in the Host
Natasa Strbo and Eckhard R. Podack (FASEB, Orlando 2001)
CD30-Ligand Deficient Mice are Resistant to Experimental Allergic Encephalitis (EAE)
Hiromi Muta*, Gisela Caceres*, P. June Ohata and Eckhard R. Podack (FASEB, Orlando 2001)

3. **Funding applied for**

Grant title and No

Conclusions:

Secreted heat shock proteins have been shown to be highly effective tumor vaccines. They can reject established tumors through the activation of CD8 CTL. Best effects are obtained by frequent boosting.
The mechanism of CD8 CTL activation has been established. Secreted gp96-Ig is taken up by CD91 on dendritic cells. Uptake results in activation of dendritic cells and transporting pg96-bound peptides to the class I MHC presentation pathway. Activated dendritic cells activate NK cells and CD8 cells in a pathway that requires the presence of perforin and that is abrogated in perforin deficient mice. The mechanism of perforin action in this sequence is under investigation.
So what?
The results are used to implement clinical and preclinical trials using secreted gp96-Ig for lung cancer, for gynecological cancers and for HIV. In addition a grant proposal is pending to study the molecular mechanisms of gp96-Ig mediated NK and CTL expansion and contraction.

References: Dr. Podack's publications during the grant period are not bolded.

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